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# Optimizing Conditions for Cloning cDNA from Chicken Brain

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OPTIMIZING CONDITIONS FOR CLONING cDNA FROM  
CHICKEN BRAIN

A Thesis  
Presented to  
the Faculty of the Department of Biology  
Western Kentucky University  
Bowling Green, Kentucky

In Partial Fulfillment of  
the Requirements for the Degree  
Master of Science

by  
Stephen W. Clark  
January, 1994



OPTIMIZING CONDITIONS FOR CLONING cDNA FROM  
CHICKEN BRAIN

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## A c k n o w l e d g m e n t s

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## OPTIMIZING CONDITIONS FOR CLONING cDNA FROM CHICKEN BRAIN

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January, 1994

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Acoustic over stimulation can lead to hair cell loss in the auditory epithelium. Hair cell loss or damage in mammals often results in decreased auditory sensitivity which is irreversible. However for birds, hair cells which are damaged by acoustic trauma or ototoxic drugs may be replaced by regeneration.

As the first step in determining the molecular mechanisms of hair cell regeneration, gene expression techniques including cDNA synthesis and cloning were attempted first with the chicken brain. The initial attempts at making cDNA libraries were elusive; therefore, experiments were performed to identify problems and enhance the efficiency of specific steps. These steps included RNA isolation, competent cell preparation, and cell transformation. The study results suggest RNA isolations can be increased with an augmented ethanol precipitation and include a shorter protocol for competent cell preparation which increases transformation efficiency. In addition, the study results imply a possible transformation advantage for thawing competent cells at lower temperatures.

## **Introduction**

### Regeneration of the auditory system:

In the United States over 17 million people experience a loss in auditory sensitivity known as sensorineural deafness or "nerve deafness." Most of these cases result from direct damage or loss in the sensory hair cells of the cochlea's sensory epithelia. The sensory hair cells, which are a vital component in the peripheral apparatus of the auditory system, play the critical role in hearing of transducing mechanical stimuli into electrical impulses capable of transmission to the brain. The sensory epithelia consist of hair cells, glia-like supporting cells, and the efferent and afferent neuron terminals (Corwin, 1992).

It has been shown that in mammals and birds the supporting cells and hair cells of the postnatal cochlea are quiescent and that their production ceases early in embryogenesis (Ruben, 1967; Katayama and Corwin, 1989). However, it was later discovered that after acoustic-mediated cell death, hair cells in birds were able to regenerate within 10 days (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). In addition, studies involving tritiated thymidine incorporation in the regenerating avian cochlea, showed that both the hair cells and supporting cells were labeled at the sites of regeneration, suggesting either cell type could be involved in regeneration (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Subsequent observations in the lateral line of axolotl salamanders, which share analogous mechanisms of mechanoreception with birds, showed that regeneration could occur without the presence of hair

cells (Balak *et al.* 1990). Due to these observations, it has been proposed that the supporting cells serve as the progenitors of the regenerated hair cells (Balak *et al.*, 1990). However, definite identification of the progenitors of regeneration has yet to be achieved (Corwin, 1992). Nonetheless, the supporting cells, if not directly involved in regeneration, are definitely influenced by the process and may still provide insight into events leading to regeneration. Furthermore, if the supporting cells prove to be the progenitors of regeneration, the obvious basic questions will be the following. What factors are involved in the stimulation of these normally quiescent cells and what are the molecular mechanisms which govern regeneration.

To answer the above questions, the molecular technique called cDNA cloning must be optimized for the chick cochlear tissue. This technique involves the enzymatic conversion of poly (A) + mRNA into a double-strand DNA molecule, one of which, is complementary to the initial mRNA molecule; thus, the molecule is called a cDNA (Efstratiadis *et al.*, 1976; Rougeon and Mach, 1976; Williams, 1981). The cDNA's are then ligated either into a plasmid or phage vector producing recombinant DNA (Coleclough and Erlitz, 1985). The ligated vector/cDNA is then introduced into *E. coli* by either infection or transformation depending on which vector is used, phage or plasmid, respectively. A complete collection (i.e., every mRNA is represented) of these recombinant *E. coli* cells is called a cDNA library, and individually they are called cDNA clones (Williams, 1981). Once cDNA clones can be produced for a certain tissue, the cDNA clones can be used to study the gene expression for that tissue



and the factors involved in the regulation of the genes (Okayama and Berg, 1982). In addition, cDNA cloning allows other techniques, such as subtraction hybridization and differential display, to be used to determine differences in gene expression between two different tissues like regenerating and non-regenerating chochlea (Davis *et al.*, 1987; Sargent, 1987; Zopf *et al.*, 1987; Liang and Pardee, 1992)].

All of the above advantages of cDNA cloning depend on efficient cloning, such that every mRNA expressed in the tissue of interest is represented at least once by the cDNA library (Williams, 1981). Since chicken brain is relatively accessible and large amounts of RNA can be extracted from it, chicken brain was used in enhancing efficiency of cDNA cloning.

Two different approaches were initially used to make a cDNA library from chicken brain. The first approach used a directional cloning procedure from Promega, with an oligo (dT) primer-Xba adapter and EcoRI adapters (Fig. 1); the second approach was a slight modification of a method combining the advantages of the classical method with the advantages of the vector-primer method (Hu *et al.*, 1992) (Fig. 2). Neither approach resulted in the production of an efficient chick brain cDNA library. However, since creating a cDNA library involves multiple steps, with each step in the process depending upon the previous step, an optimization of each step in the production of a cDNA library was undertaken; these steps included RNA isolation, mRNA isolation, competent cell preparation, cell transformation, and cDNA synthesis.

## Materials and Methods

### Chicks

Chickens 6-10 days old were sacrificed by CO<sub>2</sub> asphyxiation and then decapitated. The brain was then removed and immediately frozen in liquid nitrogen or on dry ice.

### RNA Extractions

RNA was extracted from the chick brain by the guanidinium thiocyanate-phenol-chloroform (GTPC) method (Chomczynski and Sacchi, 1987). In addition, in an attempt to augment the RNA yields, the isopropanol isolation steps were replaced with three volumes of 100% ethanol and 0.1 volumes of 3M sodium acetate pH 5.0

### Glyoxal / Agarose RNA Gel Electrophoresis

The integrity of the RNA isolated was determined by glyoxal / agarose gel electrophoresis as described previously (McMaster and Carmichael, 1977; Thomas, 1980), except that after incubation at 50°C the RNA sample was cooled in an ice bath and no sucrose was added before electrophoresis.

### Isolation of Polyadenylated RNA

Polyadenylated RNA was isolated by passing the RNA extract through an oligo (dT) cellulose column (Aviv and Leder, 1972) using the Poly (A) Quick™ mRNA purification kit (Stratagene, 1990). The RNA sample was heated in a 65°C water bath for 5 minutes. The RNA sample was then placed on ice, and an appropriate amount of 10

X sample buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5.0 M NaCl] was added for a 1 X final concentration. The sample (600 µl ) was then added to the column and pushed through at a rate of approximately one drop every two seconds (1 drop/2 sec). The sample effluent was collected and pushed through the column (1 drop/2 sec). 600 µl of high salt buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl] was applied to the column and pushed through (1 drop/sec). The high salt buffer wash was repeated three more times (600 µl). mRNA was eluted using three 200 µl aliquots of elution buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] preheated to 65°C (1 drop/sec).

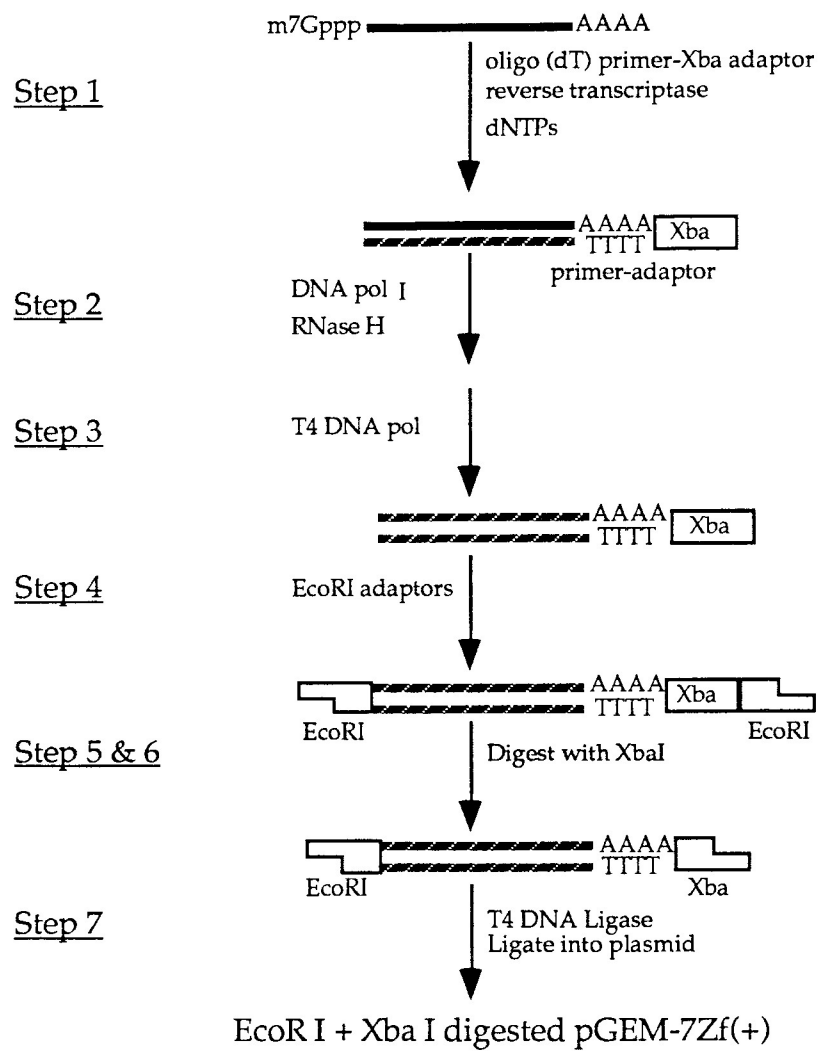
#### cDNA Synthesis with Promega's Riboclone system

cDNA's were synthesized using a cDNA synthesis kit from Promega and mRNA isolated from chicken brain according to the protocol in *Promega Protocols and Applications Guide* (1991), as shown in Figure 1. Poly A (+) RNA was hybridized with an Oligo (dT) primer-Xba I adapter and first strand synthesis was catalyzed with AMV reverse transcriptase and dNTP's (Fig. 1, step 1). Second strand replacement synthesis directly followed with RNase H and DNA pol I (Fig. 1, step 2). Synthesis was completed with the addition of T4 DNA polymerase to flush the ends of the cDNA molecule (Fig. 1, step 3).

#### Annealing the EcoR I Adapters

The formation of the EcoR I-Xmn I blunt end duplex adapters were carried out as suggested by the manufacture's guidelines (NEB, 1991).

Figure 1. Orientation-specific cloning using the primer-adapter cDNA method. mRNA is hybridized with a oligo (dT) primer-Xba adapter and first-strand synthesis is carried out with reverse transcriptase (Step 1). Second-strand cDNA synthesis is carried out with DNA polymerase I and RNase H (Step 2) and the cDNA is blunt ended with T4 DNA polymerase (Step 3). EcoRI adapters are added to both ends of the cDNA (Step 4). The cDNA is digested with Xba I and 5' phosphates are added with T4 polynucleotide kinase (Step 5 & 6). The cDNA is ligated in the digested plasmid in a orientation- specific manner with T4 DNA ligase (Step 7) (Promega, 1991).



### Addition of the EcoR I Adapters

The EcoR I adapters were ligated to the blunt ended cDNA's according to the protocol in *Promega's Protocols and Applications Guide* (1991)(Fig. 1, steps 4,5, & 6).

### Removal of Unligated Adapters

The reaction mixture was then extracted with phenol-chloroform to remove any remaining protein. To remove the unligated adapters, ammonium acetate was added to the cDNA reaction to a 2M final concentration followed by the addition of two volumes of 100% ethanol, mixed and placed at -70°C for two hours. The reaction mixture was then microfuged at room temperature for five minutes, the ethanol was removed, and the reaction mix was resuspended in TE buffer. This process was repeated, and after the second microfugation the cDNA pellet was washed with 500 µl of 70% ethanol and microfuged. The ethanol was removed and the pellet was dried under a speed vac; the cDNA pellet was then resuspended in 20 µl of TE.

### Isolation of pGEM-7Zf (+)

After growing up transformed JM109 cells, the plasmid pGEM-7Zf(+) was isolated as described previously by Maniatis *et al.* (1982).

### Preparation of pGEM-7Zf (+)

The plasmid pGEM-7Zf(+) was digested with the restriction enzymes EcoR I and Xba I as suggested by Promega (1991), except the reaction was incubated overnight at 37°C and the enzyme

concentration was 30 µg of plasmid DNA (Fig. 2). After digestion the enzymes were heat inactivated at 70°C for 15 minutes and cooled on ice for 10 minutes. The 5' phosphate was removed from the plasmid with calf intestinal alkaline phosphatase (NEB) as described by Promega (1991). The reaction was stopped with the addition of 5 mM EDTA and heat inactivated at 75°C for 10 minutes. After heat inactivation, a phenol-chloroform and chloroform wash followed. The plasmid was precipitated overnight in three volumes of ethanol and 0.1 volume of 3M sodium acetate pH 5.0.

#### Ligation of cDNA into pGEM-7Zf (+)

The ligation of the cDNA and pGEM-7Zf (+) plasmid followed the protocol described in Promega (1991), except 400 units of T4 DNA ligase were used and the reaction was incubated at 13°C overnight. In this ligation reaction a 3:1 molar ratio of vector:insert was maintained (Fig. 1, step 7).

#### cDNA Synthesis Using pGEM-7Zf(+) as the Vector-Primer

To make the vector-primer, 28 µg of pGEM-7Zf(+) were digested with 140 units of Sac I in a 100 µl reaction, containing 10 µl of 10x restriction buffer, for 1 hour at 37°C (Fig. 3, step 1). The DNA was extracted once with an equal volume of phenol-chloroform and once with an equal volume of chloroform. The DNA was precipitated overnight in 1/10 volume of 3M sodium acetate and 2 volumes of 100% ethanol at -20°C (Hu *et al.*, 1992). After centrifugation (12,000 X g for 30 minutes) the pellet was washed with 70% ethanol, dried and dissolved in 48 µl of water. Poly (dT) tails were then added to

Figure 2. Vector circle map of pGEM-7Zf(+). The closed box shows the unique restriction sites within the lac Z gene that are used in cloning and the orientation of the RNA polymerase promoters (Promega, 1991).



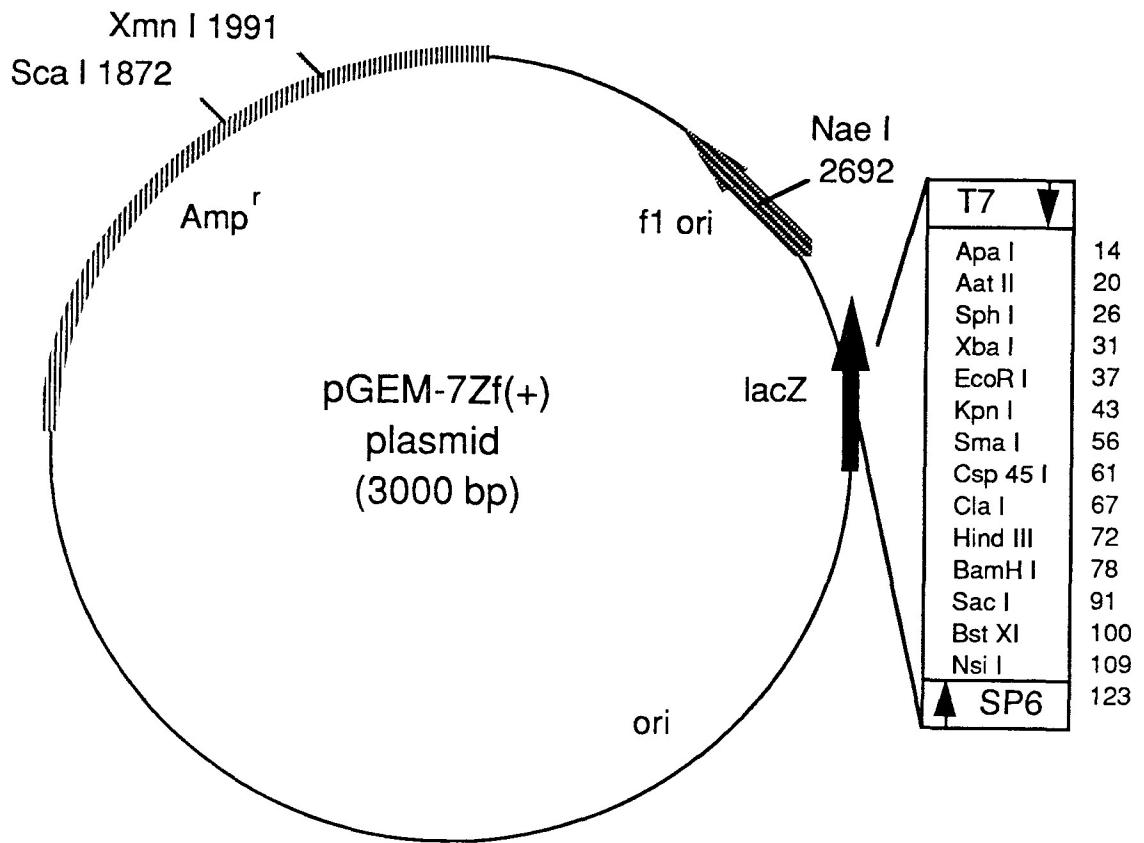
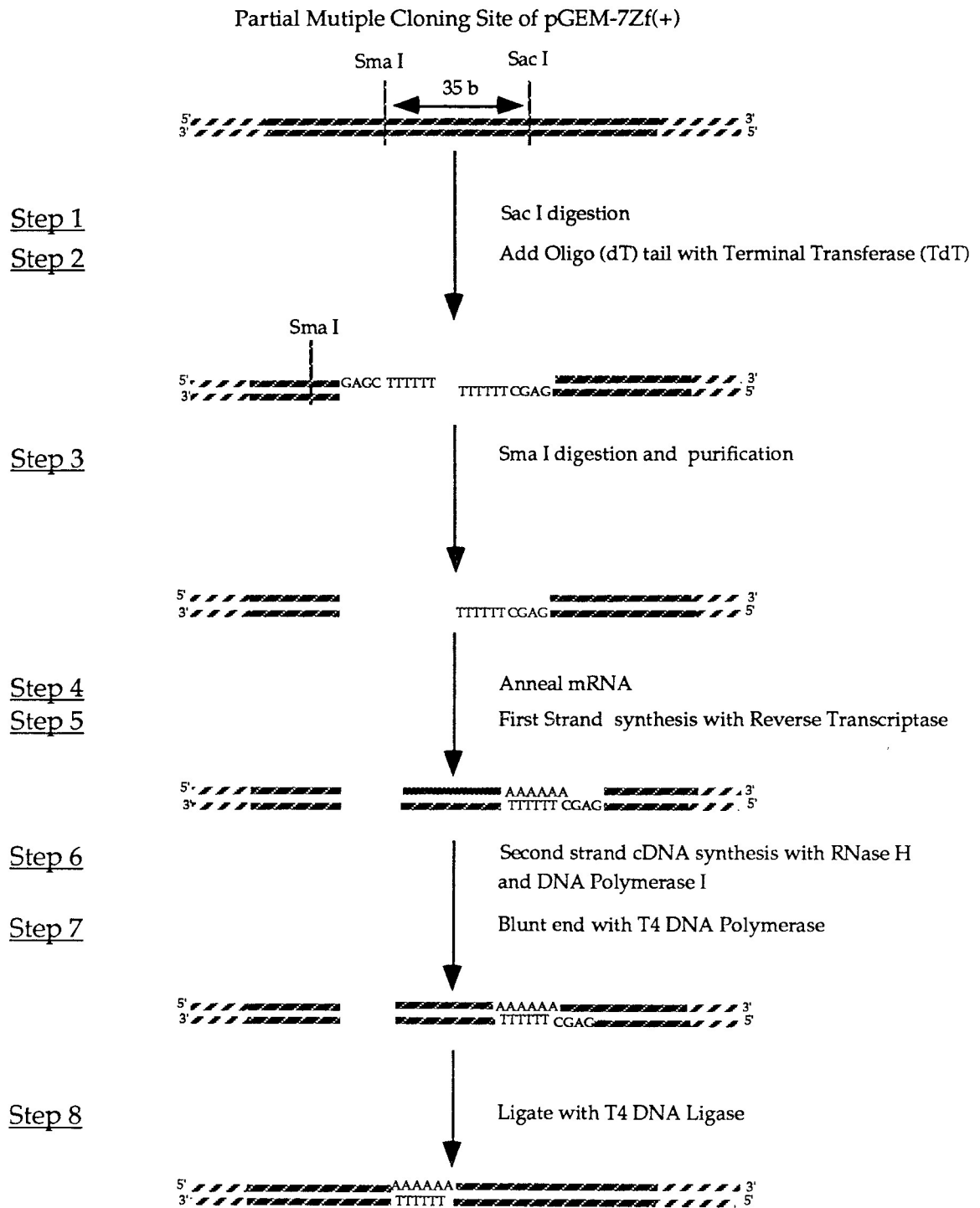


Figure 3. Diagram of efficient method for cDNA library construction with pGEM-7Zf(+). pGEM is digested with Sac I (Step 1) and an oligo (dT) tail is added to each 3' end with terminal transferase (Step 2). The plasmid is then digested with Sma I and purified from the smaller fragment (Step 3). mRNA is annealed to the remaining oligo (dT) tail (Step 4) and first strand synthesis is performed with reverse transcriptase (Step 5). Second strand synthesis is produced with RNase H and DNA polymerase I (Step 6) and the molecule is blunt ended with T4 DNA polymerase (Step 7). The plasmid is then ligated together with T4 DNA ligase (Step 8) (Hu *et al.*, 1992)



both ends of 10 µg of pGEM by incubation with 40.5 units of terminal transferase (TdT), 5x TdT buffer, for 15 minutes at 37°C (Fig. 3, step 2). The vector was then extracted and precipitated as stated above. To remove the poly (dT) tail the plasmid was digested with the enzyme Sma I (48 units of Sma I) in a 25 µl reaction for 2 hours at 37°C (Fig. 3, step 3). The vector-primer was then separated from the smaller (dT) molecule by electroelution [See "Electroelution of pGEM-7Zf(+)"], and extracted and precipitated as described above. The eluted vector-primer was hybridized to poly A (+) RNA, and first and second strand synthesis was carried out using the same protocol and enzymes as the Riboclone system (Fig. 3, steps 4-6). The vector-primer was then blunt ended with T4 DNA polymerase (Promega, 1991)(Fig. 3, step 7), and the flushed ends were ligated together as described above (Fig. 3, step 8).

#### Electroelution of pGEM-7Zf(+)

To purify the vector-primer plasmid from the small fragment generated after Sma I digestion, electroelution was performed (McDonnell *et al.*, 1977; Hu *et al.*, 1992). The Sma I digested pGEM-7Zf(+) plasmid was loaded on a 1% agarose gel containing ethidium bromide and electrophoretically separated at 4°C. The vector-primer band was removed from the gel and placed in dialysis tubing filled with 0.5X TBE. The tied dialysis tubing was then immersed in a shallow layer of 0.5X TBE and electrophoresed at 100 volts for three hours at 4°C. The polarity of the current was then reversed for two minutes to release the vector from the wall of the dialysis tubing. The buffer surrounding the gel slice was then carefully removed and

the dialysis bag was rinsed with a small quantity of 0.5X TBE. The buffer containing the vector was extracted once with equal volume of phenol/chloroform and once with chloroform and precipitated overnight at -20°C with a tenth volume 3M sodium acetate and two volumes of chilled (-70°C) 100% ethanol.

#### Promega's Competent Cell Protocol (PCCP)

The *E. coli* cell line JM109 were made competent by using a standard protocol (Promega, 1991). A competent cell preparation was started by adding 0.4 ml of an overnight culture to 40 ml of LB broth in a 250 ml flask. The cells were incubated at 37°C at 200 rpm's until an A<sub>600</sub> of 0.55 was reached. The cells were then incubated in ice water for two hours. The cells were pelleted at 2,500 xg for 15 minutes at 4°C. The pellet was resuspended in 10 ml of ice-cold sterile trituration buffer (TB<sub>1</sub>; 10 mM Pipes pH 6.8, 100 mM CaCl<sub>2</sub>, 70 mM MgCl<sub>2</sub>) and then diluted to a final volume of 40 ml with TB<sub>1</sub>. The cells were incubated on ice for 45 minutes and pelleted at 1,800 xg for 10 minutes at 4°C. The pellet was again resuspended in 4 ml of ice-cold TB<sub>1</sub> and DMSO was added to a final concentration of 7%. The cells were aliquoted in one ml volumes and frozen in 95% ethanol cooled to -70°C.

#### Modified and Shortened Competent Cell Protocol (MSCCP)

The *E. coli* cell line JM109 were made competent by a slight modification of the methods of Hanahan (1983), Inoue *et al.* (1990) and Promega's protocol (1991). Several JM109 colonies were picked from M9 plates at 4°C and used to inoculate 200 ml of SOB (Table

3)(Hanahan, 1983) medium in a one liter flask. This inoculated medium was grown to an  $A_{600}$  of 0.6 in a 25°C incubator with vigorous shaking (250 rpm's). The cells were then removed from the incubator and placed in an ice bath for 10 minutes. The cells were pelleted by centrifugation at 2,500 x g for 15 minutes at 4°C. The pellet was resuspended in 20 ml of TB<sub>2</sub> (10 mM Pipes pH 6.7, 15 mM CaCl<sub>2</sub>, 55 mM MnCl<sub>2</sub>, 250 mM KCl)(Inoue *et al.*, 1990) and then diluted up to 64 ml with TB<sub>2</sub>. The cell suspension was again incubated in an ice bath for 10 minutes, and the cells were pelleted by centrifugation at 1,800 x g for 10 minutes at 4°C. The pellet was gently resuspended in 20 ml of TB<sub>2</sub>, and then DMSO was added to a final concentration of 7%. After an additional incubation in an ice bath for 10 minutes, the cells were frozen in one milliliter aliquots, by immersion into 95% ethanol cooled to -70°C.

#### Ausubel's (Standard) Transformation Protocol for JM109 Cells

One sterile glass culture tube was chilled on ice. Ten nanograms of recombinant pGEM were then added to the culture tube in a volume from one to ten µl. Competent JM109 cells were then rapidly thawed between fingers and 200 µl were placed into the culture tube containing the recombinant pGEM. The transformation mix was incubated on ice for 10 minutes and then heat shocked by placing the tube in a 42°C water bath for two minutes. The culture tube was then placed back on ice and one ml of LB broth was added. The transformation mix was then mixed and incubated in a 37°C shaking water bath (200 rpm's) for one hour. An appropriate aliquot of this transformation mixture (10 -30 ul) was then plated on

LB plates containing 50 µg/ml ampicillin and incubated overnight at 37°C (Ausubel *et al.*, 1992).

#### Modified (New) Transformation Protocol for JM109 Cells

Competent JM109 cells were transformed with recombinant pGEM-7Zf (+) by a modification of a method as described by Inoue *et al.* (1990). One aliquot of competent cells was thawed in an ice bath, and from 100 to 800 µl of cells were dispensed into one prechilled 15 ml polypropylene tube and placed in an ice bath. Ten nanograms of recombinant pGEM-7Zf (+) were added to the competent cells in a volume from one to ten µl, and the cells were slightly shaken and placed in an ice bath for 30 min. The cells were heat shocked by placing the cells in a 42°C water bath for 30 sec and then returning them to an ice bath. At this point, 800 µl of SOC media (Hanahan, 1983) were added to the reaction followed by incubation at 37°C (200 rpm's) for one hour. A portion of this transformation (10-30 µl) was then plated on LB plates containing 50 µg/ml ampicillin and incubated overnight at 37°C.

#### Blue/White Color Selection

To determine if the beta-galactosidase gene had been disrupted and thus to select for recombinant clones, the blue/white color screening method was performed (Promega, 1991). LB plates containing 50 µg/ml ampicillin were spread evenly with 20 µl of 50 mg/ml X-Gal and 100 µl of 100 mM IPTG. These components were allowed to be absorbed by incubation at 37°C for 30 min. The plates were then spread with 10-40 µl of the transformation reaction and

incubated overnight at 37°C. Recombinant (white) colonies were then counted and compared to wild type (blue) colonies.



## Results

The isolation of total cellular RNA from chicken brain was performed by the guanidinium thiocyanate-phenol-chloroform (GTPC) extraction method as described by Chomczynski and Sacchi (1987). In addition, the GTPC method was modified by replacing the isopropanol precipitation step with three volumes of 100% ethanol and a tenth volume of 3 M sodium acetate. Replacing the isopropanol step with ethanol resulted in a 3 fold increase in the amount of RNA recovered and a 4.6 fold increase in RNA yield (Table 1). In addition, the replacement with ethanol resulted in a higher RNA purity as detected by the 260/280 ratio (Table 1).

Since free nucleotides from RNA degradation can increase the absorbance reading at 260, the integrity of each RNA isolation was analyzed by agarose gel electrophoresis under denaturing conditions using glyoxal treated RNA (McMaster and Carmichael, 1977; Ausubel *et al.*, 1992). Since the major constitute of eukaryotic cellular RNA is ribosomal RNA (Lewin, 1991), 28 and 18 S ribosomal bands were expected to be present in undegraded RNA samples. Lane 2 of figure 4 shows the presence of 28 and 18S bands for the RNA sample prepared by the modified GTPC method.

The effects of RNA purity, integrity, and RNA concentration on the percentage of mRNA collected by an oligo (dT) cellulose column was studied. In RNA sample 1 where the RNA integrity was high, the mRNA collected relative to the RNA added (mRNA/RNA) to the column was 2.9%. In contrast, RNA sample 2 had low RNA integrity and a 45% decrease in the mRNA/RNA percentage relative

Table 1. Comparing the RNA yield of different guanidinium thiocyanate-phenol-chloroform RNA isolation methods. RNA was isolated from chicken brain (1 gram of tissue) by the Chomczynski and Sacchi (1987)(GTPC) method and by replacing the isopropanol steps in the GTPC method with three volumes of ethanol. The RNA quantity, yield and purity as determined by the absorbance readings at 260 and 280 nm where compared for each method.

RNA Sample	GTPC Method I=Isopropanol E=Ethanol	RNA Recovered ( $\mu$ g)	RNA Yield (mg/g of tissue)	RNA Purity (260/280)
1	I	342	0.37	1.13
2	I	259	0.5	1.45
3	I	708	0.3	1.2
Avg.	I	436	0.39	1.26
4	E	1615	1.52	1.73
5	E	1052	0.96	2
6	E	1072	0.98	1.7
Avg.	E	1246	1.81	1.81

Figure 4. Glyoxal gel showing RNA extracted with the modified GTPC method. Lambda DNA (Lane 1) and RNA extracted with the modified GTPC method (Lane 2) were run on a 1% glyoxal gel.



to RNA sample 1 (Table 2). However, the concentration of the RNA added to the oligo (dT) cellulose column had no apparent effect on the mRNA/RNA percentage (Table 2).

Figure 5 shows an elution profile of RNA sample 6 with an  $A_{260}$  of 0.05 for the fourth high salt buffer wash and an  $A_{260}$  of 0.28 for the mRNA elution. Gel electrophoresis of the eluted mRNA revealed a range of sizes between 9.5 kb and 0.5 kb, with slightly visible 28 and 18S ribosomal RNA bands present (Fig. 6).

In an attempt to obtain competent cells that elicit high transformation frequency, several competent cell preparations were assessed (Table 3). Cell preparations 1 and 2 differed only in their  $A_{600}$  absorbance, with both being grown at 37°C with TB<sub>1</sub> trituration buffer, LB growth buffer, Promega's competent cell protocol (PCCP) (Promega, 1991), and with a transformation average of  $1.55 \times 10^6$  cfu/μg using the standard transformation protocol (Std). Cell preparation 3 was identical to preparations 1 and 2; however, steps in the PCCP protocol were modified and shortened (MSCCP), which had a 1.4 fold increase in transformation efficiency over preps 1 and 2 using the standard transformation protocol. Preparations 4 and 5 had transformation efficiencies of  $1.2 \times 10^6$  and  $1.7 \times 10^6$  cfu/μg, respectively, and were similar to preparation 3 except the trituration buffer TB<sub>2</sub> (Inoue *et al.*, 1990) replaced TB<sub>1</sub>. Cell preparation 6 had a transformation efficiency of  $3.2 \times 10^6$  cfu/μg and was similar to 4 and 5, however its growth temperature was reduced to 25°C. Cell preparations 7 and 8 had transformation efficiencies of  $7.6 \times 10^6$  and  $1.4 \times 10^7$  cfu/μg, respectively, and were identical to preparation 6, except, LB growth buffer was replaced with SOB (Hanahan, 1983).

Table 2. Effects of RNA purity, integrity, and concentration on mRNA elution from oligo (dT) cellulose column. The effects monitored were: total RNA added to the column, the RNA concentration added, RNA purity as determined by absorbance readings at 260 and 280 nm, and the integrity of the RNA added to the column determined by gel electrophoresis. For RNA integrity \* indicates RNA integrity was low (i.e. 28S and 18S ribosomal bands where degraded) and \*\*\* indicates that RNA integrity was high (i.e. the ribosomal bands where not degraded).

RNA Sample	RNA added ( $\mu\text{g}$ )	RNA Conc. ( $\mu\text{g}/\mu\text{l}$ )	RNA 260/280	RNA integrity (28,18S)	mRNA collected ( $\mu\text{g}$ )	% of RNA
1	196	0.98	1.13	***	5.76	2.9
4	500	2.5	1.73	*	6.72	1.3
6	500	2.14	1.7	***	14.4	2.9



Figure 5. mRNA elution profile from oligo (dT) cellulose column. For mRNA isolation, the RNA sample #6 was added to the column in a 600  $\mu$ l volume. The RNA sample effluent was again passed through the column and collected (Flow Thru RNA). The column was then washed four times with 600  $\mu$ l of high salt buffer and each wash elution was collected. The mRNA was eluted using three 200  $\mu$ l aliquots of elution buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] preheated to 65°C. Absorbance values (at 260 nm) were compared for all elutions.

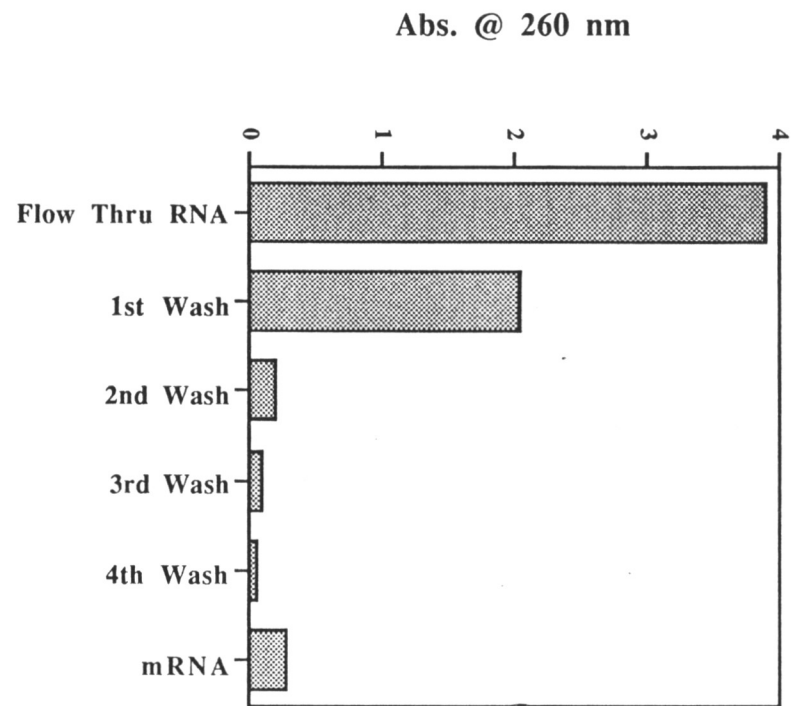


Figure 6. Glyoxal gel showing mRNA isolated with oligo (dT) cellulose column. Lambda DNA (Lane 1) and mRNA isolated with oligo (dT) cellulose column (Lane 2).

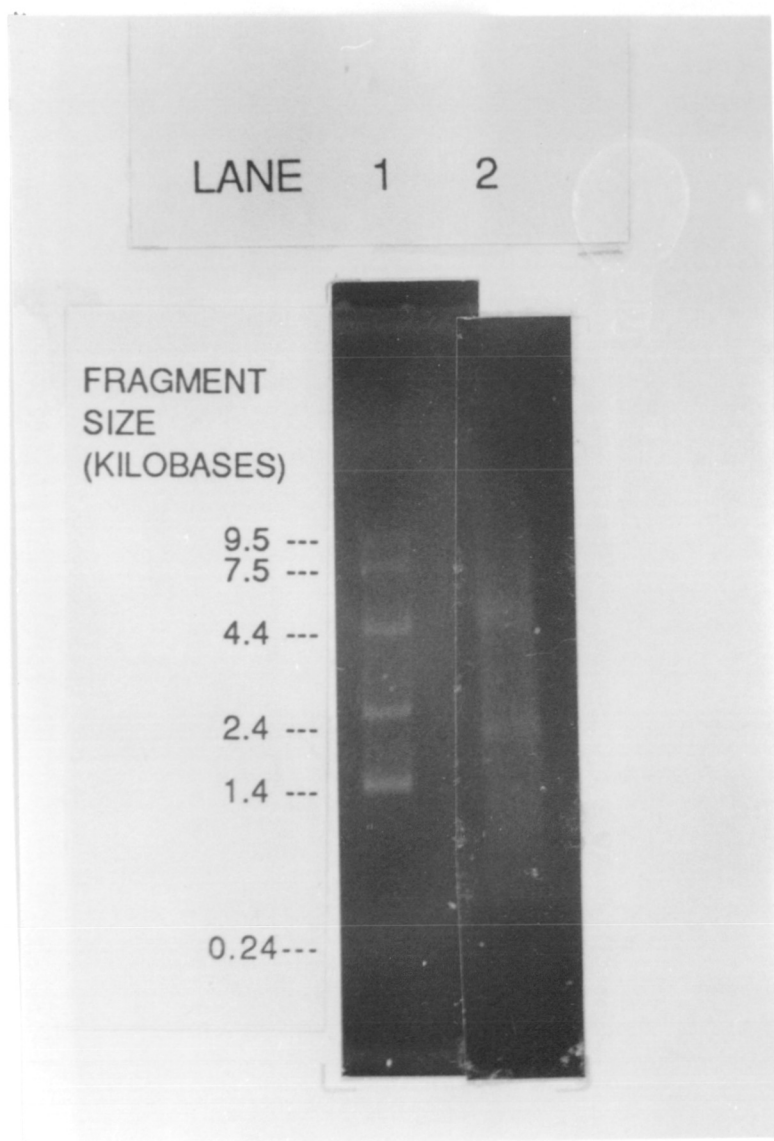


Table 3. Competent cell preparation and transformation efficiencies. Competent cells were made with different methods and materials and the merit of each preparation was determined by different transformation protocols. Abbreviations: **Cell Prep**, competent cell preparation; **A600**, absorbance at 600 nm; **Temp.**, growth temperature; **CB**, competent cell buffer; **GB**, growth buffer; **CC Prot**, competent cell protocol; **TF Prot.**, transformation protocol; **cfu/μg**, colony forming units per microgram of plasmid; **TB1**, 10 mM Pipes pH. 6.8/100 mM CaCl<sub>2</sub>/70 mM MgCl<sub>2</sub>; **TB2**, 10 mM Pipes pH. 6.7/15 mM CaCl<sub>2</sub>/55 mM MnCl<sub>2</sub>/250 mM KCl; **LB**, Luria-Bertani medium; **SOB**, 2% Bacto tryptone/0.5% yeast extract/ 10 mM NaCl/2.5 mM KCl/10 mM MgCl<sub>2</sub>/10 mM MgSO<sub>4</sub>; **PCCP**, Promega's competent cell protocol; **MSCCP**, modified and shortened competent cell protocol.

Cell Prep	A 600	Temp.	CB	GB	CC Prot.	TF Prot.	cfu/ $\mu$ g (10 <sup>6</sup> )
1	0.48	37	TB1	LB	PCCP	Std	1.6
						New	0.32
2	0.56	37	TB1	LB	PCCP	Std	1.5
						New	0.3
3	0.46	37	TB1	LB	MSCCP	Std	2.2
						New	0.5
4	0.3	37	TB2	LB	MSCCP	New	1.2
						Std	0.5
5	0.46	37	TB2	LB	MSCCP	New	1.7
						Std	0.8
6	0.58	25	TB2	LB	MSCCP	New	3.2
						Std	0.6
7	0.75	25	TB2	SOB	MSCCP	New	7.6
						Std	1.72
8	0.55	25	TB2	SOB	MSCCP	New	1.4

As described in the materials and methods, dimethyl sulfoxide (DMSO) was added to a final concentration of 7% to the competent cells prior to freezing in liquid ethanol (Inoue *et al.*, 1990). Figure 7 illustrates the stability of the competent cell preps using this protocol, with cell preparation 6 showing only a 32% decrease in competence from day two ( $3.2 \times 10^6$  cfu/ $\mu$ g) to day 79 ( $2.40 \times 10^6$  cfu/ $\mu$ g).

The assay of each cell preparation was determined by using two different transformation protocols, a standard protocol (Std) (Ausubel *et al.*, 1992) and a modified (New) protocol (Table 3); thus, cell preparations 1-7 were assessed using both the standard and modified transformation protocol, while cell preparation 8 was assayed only with the modified version. The best efficiency for each cell preparation is shown (bold) in table 3 with a range of values between  $1.5 \times 10^6$  to  $1.4 \times 10^7$  cfu/ $\mu$ g, with cell preparation 8 giving the highest efficiency.

In the standard transformation protocol, frozen competent cells are thawed rapidly at 37°C (Ausubel *et al.*, 1992). However, Inoue *et al.* (1990) suggest that competent cells be thawed at room temperature. To study the effects of thawing the competent cells on transformation efficiency, different competent cell preps were thawed at 0°C, 22°C, and 37°C (Fig. 8). In both cases, thawing cells at 0°C resulted in the highest transformation efficiency with 22°C always being the intermediate. Based on the results, illustrated in figure 8, the cells thawed at 0°C resulted in a 4 to 4.5 fold better transformation efficiency than those thawed at 37°C and about a 0.5 fold increase in transformation efficiency over 22°C.

Figure 7. The transformation stability of competent cell prep #6 when stored at  $-70^{\circ}\text{C}$ .



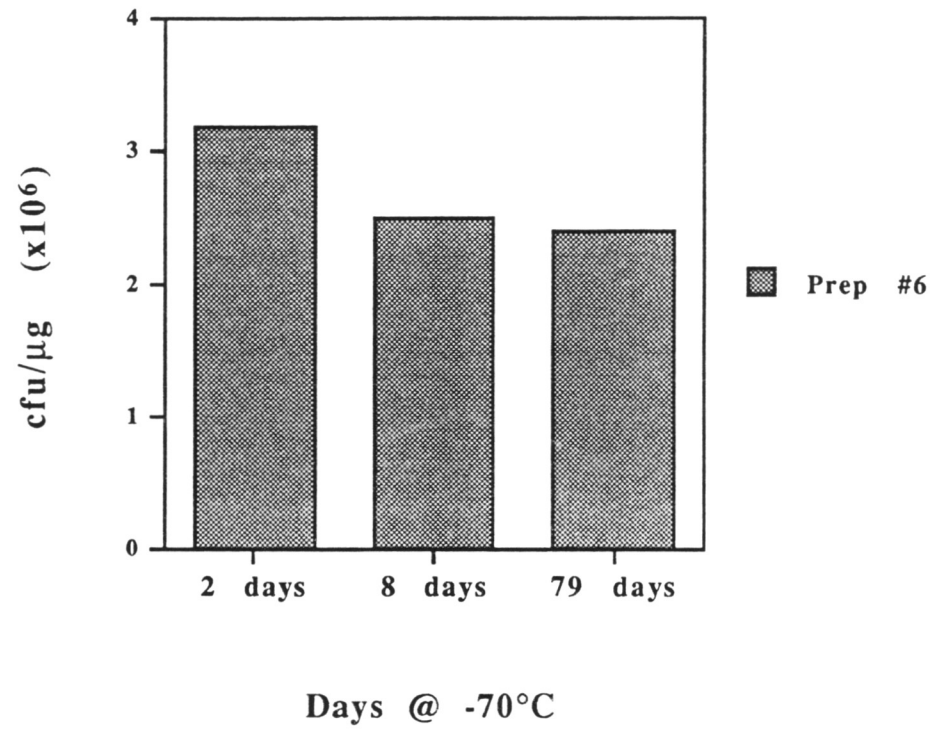
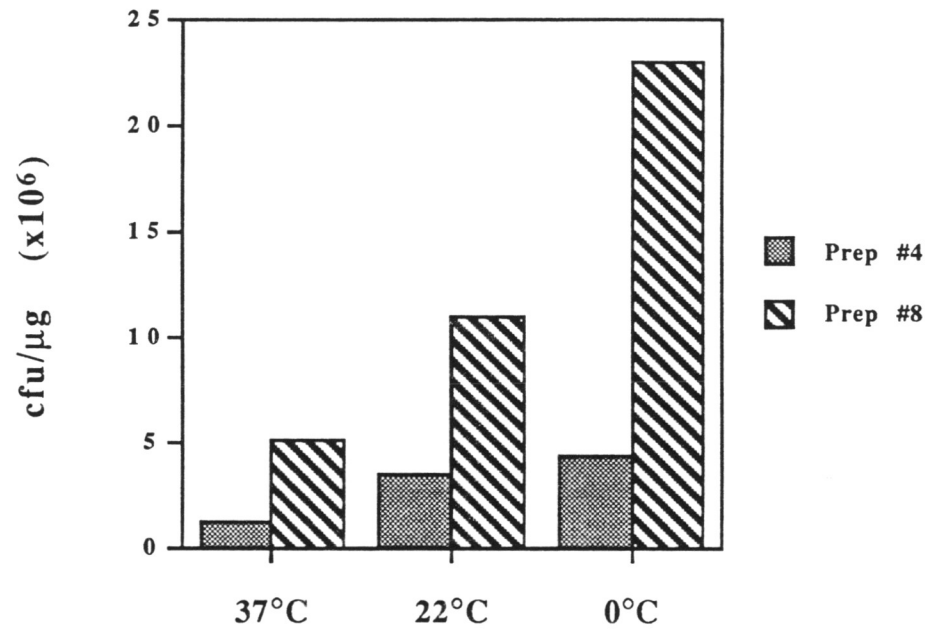


Figure 8. A comparison of transformation efficiency when using different thawing methods. The effects of different thawing temperatures on transformation efficiencies were assayed with cell preparations #4 and #8. Competent cells were removed from  $-70^{\circ}\text{C}$  and thawed either at  $37$ ,  $22$ , or  $0^{\circ}\text{C}$ .



In the standard transformation protocol (Ausubel *et al.*, 1992) glass culture tubes were used in the transformation process. According to Inoue *et al.* (1990), polypropylene tubes increase competence by tenfold over glass tubes. Thus, an experiment was performed to compare the transformation efficiency on transformations in glass and polypropylene (PPP) tubes. Transformations performed in polypropylene tubes for both cell preps 1 and 2 had approximately a threefold increase in transformation efficiency over those performed in glass (Fig. 9).

A comparison in transformation efficiencies between the standard (Std.) and the modified (New) transformation protocols is presented in figure 10. The standard transformation protocol proved to be the more efficient method for cell preparations 1-3 with an average fold increase in transformation efficiency of 4.7 over the new protocol. Cell preparations 4-7 had the highest transformation efficiency with the new transformation protocol, with an average increase in transformation efficiency of 3.8 fold over the standard transformation protocol. Cell preparation 7 responded best with a 4.4 fold increase in transformation efficiency using the new transformation protocol ( $7.6 \times 10^6$  cfu/ $\mu$ g) verses the standard ( $1.7 \times 10^6$  cfu/ $\mu$ g).

Two different techniques for generating cDNA libraries were attempted, one using the primer-adapter method and the other using a modified version of the vector-primer method as described by Okayma and Berg (1982) and Hu *et al.* (1992). When testing both methods a positive control, consisting of the plasmid pGEM-7Zf(+), was performed along with the cDNA transformations to test the

Figure 9. A comparison of transformation efficiencies when transforming in glass culture or polypropylene tubes. The effects of these tubes on transformation efficiencies were compared using competent cell preps #1 and #2.

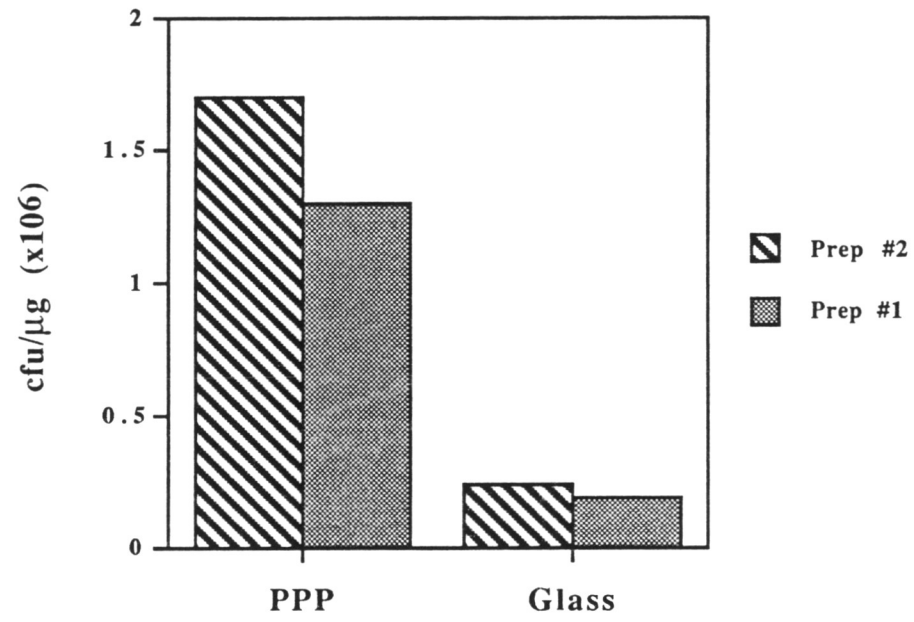
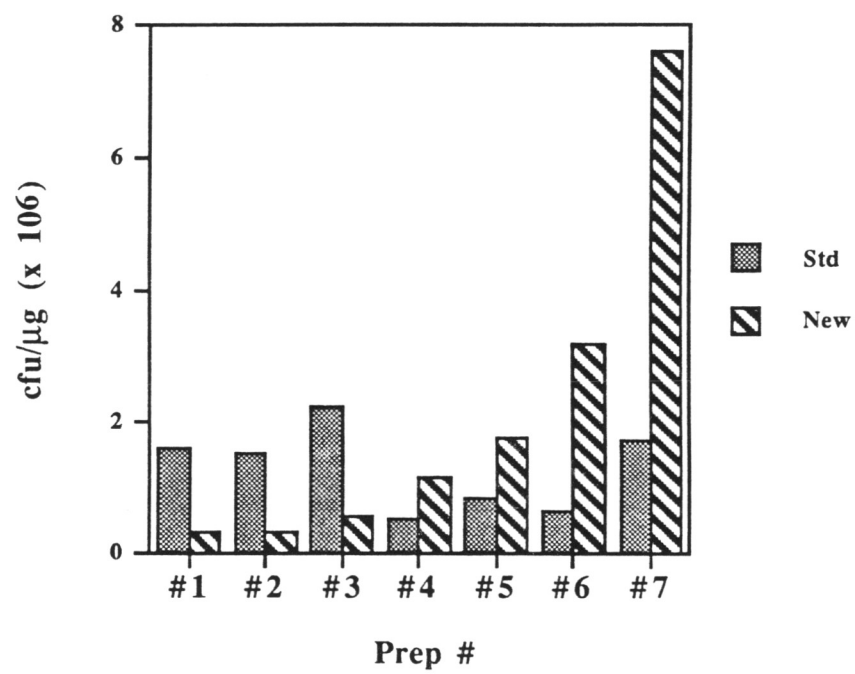


Figure 10. A comparison of transformation efficiencies using the standard (Std.) transformation method and the modified (New) method for competent cell preps 1-7.





transformation efficiency of the competent cells. Moreover, transformation efficiencies for the cDNA transformations were not calculated, since the exact amount of cDNA was unknown. For the primer-adaptor method, cDNA transformations had an average of 72 cfu/plate, and the positive control had an average transformation efficiency of  $1.3 \times 10^7$  cfu/ $\mu$ g. The same cDNA transformations were tested with the blue-white selection method and produced a 2.25% recombination average with 138 blue colonies per 3.5 white colonies. The modified vector-primer method produced an average of 57.6 cfu/plate with the positive control having an average transformation efficiency of  $4.9 \times 10^6$  cfu/ $\mu$ g. The transformations from this method were also tested with the blue-white selection method; however, no detectable recombinants were present.

## Discussion

In an attempt to augment cDNA cloning efficiency, studies were performed on RNA isolation, mRNA isolation, competent cell preparation, cell transformation, and cDNA synthesis.

Replacing the isopropanol steps in Chomczynski and Sacchi's (1987) RNA isolation method (GTPC) with three volumes of ethanol resulted in significant changes in RNA yield and RNA purity. Data in table 1 show that RNA isolated with three volumes of ethanol resulted in a threefold increase in the RNA yield over isolation with isopropanol. In addition, RNA isolated with ethanol contained less contaminating proteins as determined by the 260/280 ratio (Table 1). Figure 4 shows RNA isolation 6 with intact 28 and 18S ribosomal bands present and exhibiting a 2:1 ratio by ethidium bromide staining, thus indicating no substantial degradation of RNA isolated with the ethanol method; hence, the comparisons between the isopropanol and ethanol methods in RNA yield and purity are appropriate, and the higher 260/280 values for ethanol isolations are not due to RNA degradation. Since an alcohol becomes more hydrocarbon-like as its solubility in water decreases (Hart, 1987), a possible explanation for the advantage of ethanol over isopropanol in RNA isolations could be due to a greater miscibility of ethanol with water. Thus, ethanol could have a greater ability to replace the RNA in RNA-water hydrogen bonding and increase RNA yield and purity.

In comparing the percentage of mRNA collected between RNA samples 1, 4, and 6 in table 2, it seems that mRNA recovery is not affected by RNA purity but is affected by RNA integrity. In addition,

by comparing experiments 1 and 6, the quantity of RNA added to the oligo (dT) column has little influence on the percentage of mRNA collected.

The elution profile of experiment 6 (Fig. 5) follows exactly the pattern expected for mRNA elution (Aviv and Leder, 1972), with each subsequent high-salt buffer fraction resulting in decreasing  $A_{260}$  values and with the mRNA fraction resulting in a increasing  $A_{260}$ . However, it is questionable how much of the mRNA elution is indeed mRNA, since the mRNA gel (Fig. 6) shows considerable RNA presence with distinct 28 and 18S bands still visible.

The efforts to synthesize and clone cDNA's proved to be non-productive. As stated in the results, the primer-adapter method, with the Xba-primer and EcoRI adapters, generated only a 2.25% recombinant average, and the vector-primer method of Hu *et al.* (1992) generated no detectable recombinant clones. Unfortunately, cDNA synthesis requires a large number of steps in which each step depends on the success of the previous one for the production of a efficient cDNA library. The primer-adapter method required the addition of EcoRI adapters followed by multiple ammonium acetate washes to separate the cDNA from the unligated adapters and then ligation of the cDNA into the pGEM-7Zf(+) plasmid. Thus, the many steps involved in this attempt could have resulted in a considerable loss of cDNA. In addition, the enzymatic steps involving the ligation and restriction with Xba I could have been sources of possible problems, since many of these enzymes were several years old. The latter reason could also explain why the vector-primer method proved fruitless, especially with regard to the ligase and the addition

of the oligo (dT) tail. Equally, with the primer-adaptor method, the lack of cDNA size fractionation might have contributed to the low recombinant percentage, with small incomplete sizes of DNA competing with normal cDNAs (Promega, 1991). Furthermore, as stated above, it is uncertain how much of the mRNA elution was contaminated by rRNA; thus, if the mRNA fraction was considerably contaminated by rRNA, then quantitation of mRNA could have been significantly over estimated in the cDNA reactions. Since reverse transcriptase is known to synthesize unclonable hairpin structures at low mRNA concentrations (Stratagene, 1990), over estimation of mRNA quantity could explain the low recombinant averages. In addition, no experiments were performed with different cDNA to vector ratios, due to the fact that only small amounts of cDNA were generated; thus, in the cloning attempts the cDNA concentrations could have been too low for significant recombination (Promega, 1991).

Experiments testing the variables involved in the preparation of competent cells revealed a number of significant and interesting results. First, by comparing cell preparations 1 and 3 (Table 3) it is clear from transformation efficiencies that there is no disadvantage to using the shortened and modified competent cell protocol (SMCCP). Thus, competent cells prepared with the SMCCP protocol not only provide a much faster method of producing competent cells but also produce competent cells with a slightly better transformation efficiency. Secondly, the temperature at which the competent cells are grown seems to be paramount in producing high transformation efficiencies (Table 3, preps 7 and 8), with growth at 25°C giving a

significant increase in transformation efficiency. Inoue *et al.* (1990) state that growing *E. coli* cells at 18°C yielded the highest competence with an optimum cell density ( $A_{600}$ ) of 0.75; no attempt was made to grow the competent cells at 18°C, since Inoue *et al.* (1990) stated that 25°C gave similar results, but with a slightly lower competence.

Finally, by comparing cell preparations 7 and 8, it is apparent that for growth at 25°C, the optimum cell density ( $A_{600}$ ) is approximately 0.55. Thus, the most efficient competent cell preparation was preparation 8, using the MSCCP protocol, a growth temperature of 25°C,  $A_{600}$  of 0.55, SOB growth buffer, and TB<sub>2</sub> competent buffer. Moreover, cell preparation 6 proved relatively stable when stored at -70°C with only a 23% decrease in competence after 79 days (Fig. 7).

Even though cell preparation 8 had a transformation efficiency of  $1.4 \times 10^7$  cfu/μg, approximately a tenfold increase in the highest observed efficiency, this was roughly 100-fold less than what Inoue *et al.* (1990) had observed with the same *E. coli* strain JM109. Excluding slight modifications, there are only two significant differences in technique between what Inoue *et al.* (1990) describe and the competent cell protocol followed to produce cell preparations 7 and 8; first, the cells were not grown at 18°C but at 25°C and, secondly, the cell preps were frozen in 95% ethanol at -70°C, instead of liquid nitrogen. However, one cell prep was frozen in liquid nitrogen; the results were poor; and no further duplication was attempted. In future experiments, it would be interesting to test more cell preps with liquid nitrogen and, in addition, grow the cells at 18°C, to determine whether a competence frequency comparable to Inoue *et al.* (1990) could be achieved.

Three stages have been implicated in the transformation process: induction of competence, DNA uptake, and cell recovery (Bergmans *et al.*, 1981). Hanahan (1983) describes only two stages--DNA uptake and the establishment of the DNA as a stable genetic element. Unfortunately, the transformation results presented provide few answers to explain the biophysical and biological events involved in the transformation process. For competent cell prep's 1-3 it is apparent (Fig. 10) the standard transformation protocol is the most effective. However, for prep's 4-7 the new transformation protocol works best. The basic differences between the standard and new transformation protocols are as follows. The standard protocol is carried out in glass culture tubes whereas the new protocol is performed in a polypropylene tubes; DNA is added before the competent cells in the standard protocol and the reverse is true for the new protocol; competent cells are thawed at room temperature in the standard and at 0°C in the new protocol; and the new protocol uses SOC (Hanahan, 1983) growth medium whereas the standard uses LB. To more fully discern the transformation process, two of these differences were further studied, the use of polypropylene over glass and different thawing temperatures.

Transformations studying polypropylene versus glass culture tubes (Fig. 9) show that polypropylene use increases the transformation efficiency by threefold over glass but not by ten-fold as Inoue *et al.* (1990) suggest. Inoue *et al.* (1990) do not discuss why polypropylene works best, however, it could be that the glass tubes become positively charged and compete with the competent cells for the negatively charged DNA. This explanation might be verified by

siliconizing both the glass and polypropylene tubes and thus decreasing the ionic interactions of the glass and polypropylene with RNA.

The most intriguing results were from the different thawing methods of the competent cells (Fig. 8). Cell preparation 8 showed that there was a significant transformation advantage in thawing at 0°C compared to 37 or 22°C. From this data it is not clear whether the transformation advantage from thawing is due to an increase in competence of the cells or simply an increase in the viability of the competent cells (i.e., more competent cells survive). However, because the actual mechanisms of transformation and competence induction are so poorly understood (Taketo, 1972; Hanahan, 1983) and since the transformation process itself is probably better explained biophysically than biologically (Jones *et al.*, 1981), it is a conceivable possibility that thawing, like the freezing (Inoue *et al.*, 1990) of competent cells, may increase the competence state.

Evidence from this study supports the claim of Inoue *et al.* (1990) that in competence induction, growth temperatures lower than 37°C are preferred with *E. coli* cells. In addition, this study also supports Inoue *et al.* (1990) in the use of polypropylene over glass tubes in the transformation process. However, with both the growth temperature and in the use of polypropylene tubes, the transformation efficiencies generated were not as high as those of Inoue *et al.* (1990). Furthermore, data from this study accentuate the role of thawing in the process of competence induction and suggest that less stringent thawing methods (i.e., lower temperatures) may enhance the competence of *E. coli* cells.

From this study, it is clear that the cDNA cloning process from cDNA synthesis to induction of competence and uptake of DNA is a very complicated and multistep process in which all the steps must work to produce efficient cloning. However, it is hoped that experimental results presented in this investigation will facilitate future experiments or attempts at cDNA synthesis and library construction and accelerate the understanding of the molecular events involved in hair cell regeneration.



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